Optimized production and purification of Coxsackievirus B1 vaccine and its preclinical evaluation in a mouse model

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Highlights

- An efficient CVB1 vaccine production and purification process was developed.
- Formalin inactivation caused a dramatic change in CVB1 integrity.
- Tween80 detergent increased CVB1 yield and stability significantly.
- CVB1 vaccine was immunogenic and protected mice against CVB1 infection.

ABSTRACT

Coxsackie B viruses are among the most common enteroviruses, causing a wide range of diseases. Recent studies have also suggested that they may contribute to the development of type 1 diabetes. Vaccination would provide an effective way to prevent CVB infections, and the objective of this study was to develop an efficient vaccine production protocol for the production of novel CVB vaccines. Various steps in the production of a formalin-inactivated Coxsackievirus B1 (CVB1) vaccine were optimized including the Multiplicity Of Infection (MOI) used for virus amplification, virus cultivation time, type of cell growth medium, virus purification method and formulation of the purified virus. Safety and immunogenicity of the formalin inactivated CVB1 vaccine was characterized in a mouse model. Two of the developed methods were found to be optimal for virus purification: the first employed PEG-precipitation followed by gelatin-chromatography and sucrose cushion pelleting (three-step protocol), yielding 19 fold increase in virus concentration (0.06 µg/cm^2). The second method utilized tandem sucrose pelleting without a PEG precipitation step, yielding 83 fold increase in virus concentration (0.24 µg/cm^2), but it was more labor-intensive and cannot be efficiently scaled up. Both protocols provide radically higher virus yields compared with traditional virus purification protocols involving PEG-precipitation and sucrose gradient ultracentrifugation. Formalin inactivation of CVB1 produced a vaccine that induced a strong, virus-neutralizing antibody response in vaccinated mice, which protected against challenge with CVB1 virus. Altogether, these results provide valuable information for the development of new enterovirus vaccines.

Keywords

Coxsackievirus B1, CVB1, vaccine, virus purification, formalin inactivation

1. Introduction

Coxsackievirus B1 (CVB1) belongs to the enterovirus (EV) family and has a non-enveloped, icosahedral ~30 nm diameter capsid, consisting of 60 copies of the structural proteins VP1-4. During replication, the viral polyprotein P1 is cleaved into structural proteins VP0, VP1 and VP3. VP0 is further processed into VP2 and VP4 in a viral RNA-driven autocatalysis reaction resulting in the formation of mature viral capsids [1-3].

EVs are the most common infection causing viruses in humans. Generally EVs infect the respiratory and gastrointestinal tracts and frequently lead to viremia. Sometimes, this can result in secondary infections in organs, such as the heart or central nervous system causing for example polio, meningoencephalitis [4-7], sepsis [8], pancreatitis [9] or myocarditis [10]. Children younger than three years are the most susceptible to severe EV diseases and recent CVB1 epidemics have caused life-threatening infections in neonates [10-15].

Associations between EV infections and the development of chronic diseases such as type 1 diabetes (T1D), dilating cardiomyopathy, asthma and allergies [16] have been documented. T1D is thought to result from an immune-mediated destruction of the insulin-producing β-cells in the pancreas. Environmental triggers of T1D have been purported including CVBs (CVB1-6), and particularly CVB1 [17,18]. However, the mechanisms through which EVs may cause different disease pathogenesis are poorly understood, but persistent infections and viral proteases have been implicated [19-21].

Alongside traditional poliovirus vaccines, EV vaccines have been developed against a limited number of non-polio EV diseases. Three formalin-inactivated EV71 vaccines have been tested in phase 3 trials and are effective against hand-foot-and-mouth disease in children. Moreover two of them, have recently received regulatory approval in China [22].

In the present study, we describe an optimized and scalable production and purification protocol for CVB1 vaccine and demonstrate its ability to induce a strong, virus-neutralizing antibody response and protection against CVB1 infection in mice.

2. Materials and Methods

Virus strain, cell lines and cell culture media

A field isolate CVB1 was used [17], hereinafter referred to as CVB1 and the ATCC CVB6 strain Schmitt was employed (henceforth CVB6). CVB1 and CVB6 were propagated in green monkey kidney cells (Vero) in multilayer (Falcon Cell culture Multi-Flask or Corning hyperflask M cell culture vessel) flasks using SFM4MegaVir protein-free medium (HyClone) without fetal bovine serum (FBS). Vero-cells were cultivated in DMEM containing 10% FBS, 2 mM L-Glutamine and 1% penicillin-streptomycin. Cell culture reagents were from Thermo Fisher Scientific.

Optimization of CVB1 production and purification

Different conditions were used for virus production and batches were designated CVB1#1-4 and CVB6#1. The details of the virus production (Supplementary Table 1.) and purification (Supplementary Table 2.) protocols are described in the supplementary material.

Virus characterization

Purified viruses were run on mini-protean TGX stain-free precast gradient gels (4-20%) (BioRad). Tryptophan-containing proteins were detected in SDS-PAGE gels by UV-induced fluorescence. Subsequently, viruses were electroblotted onto a PVDF membrane and analyzed by Western blotting using mouse anti-enterovirus clone 5-D8/1 (DAKO, Glostrup, Denmark; 1:3000) for CVB1 and in-house produced rat polyclonal antibody clone 1685 for CVB6, followed by incubation with IRDye-labeled secondary antibodies (1:5000). Virus and impurities were assessed by densitometry analysis of SDS-PAGE gels using ImageJ software [23]. Fibronectin was also detected by Western Blotting using anti-fibronectin antibody (Sigma). Total protein concentrations of the viruses were determined using the Pierce BCA Protein Assay kit (Thermo Fischer Scientific). Vero cell residual DNA quantitation from the purified viruses was performed using the resDNASEQ quantitative qPCR kit (Applied Biosystems), Applied Biosystems 7500 Real-Time PCR system and 7500 system SDS software.

Dynamic light scattering (DLS) analysis was performed with a Zetasizer Nano ZS instrument (Malvern Instruments Ltd.). The hydrodynamic diameter was determined using viruses diluted 1:10 and three 10 x 10-second datasets at 25 °C. Transmission electron microscopy (TEM) analysis was performed as described [24]. Virus titers were determined in Green Monkey Kidney (GMK) cells using either $TCID_{50}$ or plaque titration assays [17].

Vaccine preparation

R-buffer in batch CVB1#2 was exchanged by dialysis to PBS-0.5 mM $MgCl_2$. Virus was inactivated in 0.01% (vol/vol) formalin for 12 days at 37°C and inactivation confirmed by the lack of infective

virus in plaque titration assay. Inactivated CVB1 was diluted in PBS-0.5 mM $MgCl_2$-0.01% formalin and stored at -70°C.

Vaccinations, virus challenge, virus titering and seroneutralization assays

NOD (Non-obese diabetic) mice [25] were bred and housed in a pathogen free environment at Karolinska Institutet (Stockholm, Sweden). Experiments were approved by the local ethics committee and conducted in accordance with the NIH Principles of Laboratory Animal Care and national laws. An extended monitoring of animal health was conducted regularly. Vaccinations were given interscapularly in 150 µl to NOD mice, starting at 4-6 weeks of age. In experiment 1, mice received 1.8 µg vaccine protein per injection on days 0, 21 and 35 and control mice received vaccine buffer alone. Serum was collected on days 0, 21, 35, 42 and 49 by tail bleeding or terminal heart puncture (day 49) and neutralizing antibodies against CVB1 were detected as described [26]. Mice in experiment 2 received either 1 µg or 10 µg CVB1 vaccine on days 0, 14 (both) and 28 (1 µg only) and were challenged with 10^6 PFU CVB1 10796 [27] by intraperitoneal injection on day 35 (un-vaccinated littermates controls were also challenged). Serum samples were collected as before on days 0, 14 and 28 for the measurement of neutralizing antibody titers. Blood and pancreas were collected on days 38 and 40 respectively for the measurement of virus by standard plaque assay and the detection of viral VP1 protein by immunohistochemistry [28]. Statistical significance was determined by one-way ANOVA using GraphPad Prism version 5.

3. Results

Effect of cultivation conditions on virus yields

The Vero cell-line was chosen as a preferred CVB1 production host, since it is widely accepted by regulatory authorities and has been used in poliovirus vaccine production for over 30 years [29]. In order to prepare CVB1 vaccine free of animal derived contaminants, SFM4MegaVir (Hyclone) cell culture medium optimized for virus amplification was chosen. The effect of MOI and culture time on CVB1 production in Vero-cells was examined (Supplementary Figure 1). Virus yields did not differ significantly between time points with MOI 0.01 and therefore, MOI 0.01 and a cultivation time of 7 DPI were selected as preferred cultivation conditions (longer incubation times guarantee strong cytopathic effect and cell detachment).

Optimization of CVB1 purification

We compared five different purification protocols (outlined in Figure 1). Analysis of purified virus by SDS-page showed CVB1#1 and CVB1#2 had a high purity (~99% and ~90% respectively) and highlighted the presence of proteins approximately 31, 29 and 26 kDa in size (Figure 2A) which correspond to the molecular weights of the capsid proteins VP1-3. VP1 was also recognized by Western Blotting (Figure 2B). When virus cultivation time was increased to seven days, the purity decreased (CVB1#3a was ~66% pure, as estimated by densitometry) compared to CVB1#2 (2 day cultivation). Mass spectrometry analysis identified 260 kDa band to represent fibronectin-1 (Figure 2A), which was found to be the most prevalent contaminant in CVB1#3a (derived from Vero cells, data not shown). The Vero cell residual DNA quantity of the purified viruses was analyzed by qPCR. The DNA content was found to be very low and it varied between 0.0005 – 0.54 ng/µl in the finally

formulated virus or vaccine preparations (Supplementary Table 3). In the vaccine used in our experiments the amount of residual host DNA was 1 ng/dose, which is under the limit (10 ng/dose) for human parenteral vaccines given by WHO [30].

The tandem pelleting protocol used for CVB1#2 and CVB1#3a (Figure 1) is quite laborious, as the initial virus concentration step also used ultracentrifugation. To streamline the protocol for CVB1#3b, we utilized a combination of PEG precipitation and 30/50% sucrose cushion pelleting (Figure 1: two-step protocol). However, SDS-page and western blotting analyses identified fibronectin as the most prominent protein purified in CVB1#3b (Figure 2A) with the virus purity estimated to be ~35% by densitometry analysis. Virus recovery from CVB1#3a was 7X higher compared with the otherwise identical batch CVB1#3b, indicating a loss of virus in the PEG-precipitation step. Nevertheless, PEG-precipitation is a simple and easily scalable method for concentrating virus and also acts as a purification step. Since CVB1#3b appeared to be otherwise free of contaminants except for fibronectin, we used gelatin affinity chromatography to remove fibronectin [31]. CVB1#4, was PEG-precipitated, incubated with gelatin resin and pelleted by 30/50% sucrose cushion ultracentrifugation (Figure 1: three-step protocol) and resulted in ~95% pure virus (Figure 2A). In order to demonstrate that this aforementioned three-step protocol is applicable for the purification of other enteroviruses, we also used this protocol for CVB6 purification and the result was in line with CVB1 (Figure 2C and D).

The highest virus yield and infectivity titer was found in CVB1#3a (tandem pelleting protocol in the presence of 0.1% Tween80; Figure 2E), with the yield being 83 times higher than the CVB1#1 yield (purified with the conventional enterovirus purification method). Virus yields for CVB1#1-4 were 3, 26, 241, 35 and 56 ng/cm^2 (cultivation area) respectively, whereas their infectivity titers were 8.5 x 10^5, 1.6 x 10^6, 9.8 x 10^6, 6.7 x 10^5 and 1.1 x 10^6 $TCID_{50}$ units/cm^2. CVB6 had a virus yield of 124 ng/cm^2 and infectivity titer 4.1 x 10^5 $TCID_{50}$ units/cm^2. Therefore, both concentration and titer measurements indicated that PEG precipitation results in substantial virus loss (Figure 2E).

The quality of the purified CVB1#1–4 and CVB6#1 was determined with DLS (Figure 2F), and average sizes of the virus particles were 32 – 45 nm (Figure 2G). Due to the low concentration of

CVB1#1, the major DLS peak represents Tween micelles with size of 10 nm. In summary, DLS analysis showed all of the batches contained intact virus particles.

Effect of buffers on the stability and homogeneity of the virus

To enable optimal CVB1 purification, we evaluated the effect of buffer composition on virus stability and homogeneity. An important indicator of virus stability is particle size, as viruses may aggregate or dissociate into subunits during storage which can be monitored by changes in particle size by DLS. CVB1 particle size was studied in four buffers; R-buffer (pH 7.5) and citrate buffer (pH 5) are used in chromatographic purification techniques, whereas PBS-Tween (pH 7.4) and M199-Tween (pH 7.4) are suitable for vaccination. Virus stored at 4°C was measured by DLS on days 0 and 7 (Figure 3). In R-buffer, the particle diameter size (37.5/34.4 nm on days 0/7) and sample polydispersity index (PdI; 0.431/0.476 days 0/7 respectively) remained constant (Figure 3A). CVB1 virus showed a wider particle size range in citrate buffer (32.9/52.6 nm), but the sample was relatively monodisperse (PdI: 0.256/0.221; Figure 3B), indicating that it might aggregate during storage in acidic buffer with a low osmolality. The CVB1 batch solubilized in PBS-0.1% Tween was very homogenous (PdI: 0.234/0.230) and contained a 100% population of particles sized 46.6 nm and 54.7 nm on days 0 and 7 respectively (Figure 3C). M199-0.1% Tween solubilized CVB1 was also very homogenous (PdI: 0.347/0.247) containing only particles with size 41.6 nm (d 0) and 40.0 nm (d 7; Figure 3D). The results highlight the suitability of M199 buffer, which is widely used in poliovirus vaccines, for formulating and storing CVB1 viruses as well.

Vaccine preparation and characterization

We next tested the immunogenicity of the purified virus preparations. CVB1#2 was completely inactivated with formalin to produce vaccine [32] (virus titers dropped from 1.2 x 10^{10} PFU/ml to 0 PFU/ml). However, formalin inactivation affected the virus structure and concentration. The light scattering intensity decreased 4.6X after formalin inactivation indicating a decrease in concentration. Light scattering intensity analysis showed that before the inactivation step, the derived count rate of the CVB1#2 was 7969 kcps and after the inactivation step, the scattering intensity had decreased to 1713 kcps and therefore 78.5% of the virus had dispersed during the inactivation step (Figure 4A). Also, according to the SDS-PAGE and VP1-protein quantification by Western blot, the virus concentration dropped from 0.317 mg/ml before inactivation to 0.0165 mg/ml (5% recovered) after inactivation (Figure 4B). TEM analysis revealed a dramatic change in the morphology of the inactivated vaccine. Most of the virus was intact before inactivation, showing icosahedral full (white) and empty (grey) particles (Figure 4C) whereas after inactivation, the preparation had a heterogeneous and blurred appearance and only rarely were intact particles detected (Figure 4D).

CVB1 vaccine is immunogenic and protects mice against CVB1 infection

The immunogenicity of the CVB1 vaccine was tested in mice by injecting 3 x 1.8 µg non-adjuvanted vaccine to two mice. Sera from CVB1 immunized mice was evaluated for CVB1 neutralizing ability in vitro. Both vaccinated mice generated neutralizing antibodies at day 21 (after 1 vaccination) with titer 64 and at day 35 (after two vaccinations) the neutralizing titer was ≥1024 (Table 1).

In the second vaccination experiment, mice received either 3 x 1 µg or 2 x 10 µg non-adjuvanted vaccine which had no adverse effects on weight gain (Figure 5A) in either group. Mice receiving 10 µg vaccine showed some signs of inflammation around the injection site, hence only 2 vaccinations

were performed. All vaccinated mice mounted a strong neutralizing antibody response compared to controls ($p<0.001$) and similar to those seen in the first experiment (Figure 5B). On day 35, the vaccinated animals and littermate controls were challenged with 10^6 PFU CVB1 to examine the protective efficacy of the vaccine against infection. All mice challenged with CVB1 showed weight loss after CVB1 infection (Figure 5C). Three out of four control mice had viraemia on day 3 post infection whereas no replicating virus was detected in the blood of the vaccinated animals (0/7; Figure 5D; $p<0.05$). Furthermore, replicating virus was also measured in the pancreas of 3/4 control mice on day 5 post infection but in none of the vaccinated mice (Figure 5E). This was also confirmed by immunohistochemical analysis with VP1 positivity seen in the pancreas of 3/4 control mice but none of the vaccinated animals (representative images Figure 5F). These results indicate the vaccine protected against acute CVB1 infections in NOD mice.

4. Discussion

For the production of new vaccines, the physicochemical and immunological properties of a virus need to be carefully characterized and standardized. In the enterovirus vaccine field, the production, purification, formulation and analysis protocols have been standardized for polioviruses [33,34]. However, very limited information is available regarding the effect of formalin-inactivation on the quality and morphology of enterovirus particles despite the fact this is the method of choice for inactivating and introducing new enterovirus vaccines (including EV71 vaccines [35]) to the market. In this study, our objective was to develop a scalable production and purification method for a CVB1 vaccine with thorough characterization in order to facilitate the safe development of CVB1 vaccines.

To avoid animal derived contamination of the vaccine, serum free production medium was used for cell cultivation. Additionally, Vero cells were chosen for virus propagation due to their previous acceptance by regulatory authorities for human vaccine production [29]. Previously, poliovirus and EV71 were shown to grow well in Vero cells [34,36], whereas CAV6 and CAV10 grew poorly [37]. In

our study, CVB1 grew consistently well in Vero cells but modified Vero cell lines may further increase yields as described [38].

We restricted the demand on virus seed by applying a low MOI strategy with longer incubation times for virus propagation. During long virus cultivation periods, virus production was amplified with final virus yields comparable to those with short cultivation times and higher MOIs. Similar low MOI strategies have been described in the efficient propagation of EV71 [36,39] and poliovirus [34].

Stability of CVB1 in different buffers was examined to establish whether variation exists in conditions such as low salt and acidic pH (required for chromatographic purification methods); to compare to the R-buffer previously used in purifications [24]; and using buffers suitable for use in murine and human vaccines. These results were encouraging, as CVB1 was stable for one week in all buffers tested although the buffer containing low salt and low pH (pH 5), had the most significant particle size shift. Accordingly, we identify PBS or M199 media (pH 7.4) containing 0.1% Tween80 as the preferred buffers for CVB1 formulation, due to the Tween80 induced increase in virus yield and stability. This most likely occurs due to Tween80 forming complexes with CVB1 particles which increase recovery from the production medium by protecting the virus from unspecific binding to cell debris and plastic surfaces during purification. Of the two, M199 is the most preferable as it is already used in poliovirus vaccine [40] and accepted for human use, plus it contains a number of potentially virus stabilizing components.

Another important feature of vaccine development is an easily standardized purification method, which is suitable for scaling-up. The gold standard for enterovirus purification is based on PEG precipitation followed by sucrose gradient ultracentrifugation and final concentrating by further ultracentrifugation. According to the literature [24,37] and our own results, this procedure leads to very pure virus but extremely low yields (Figure 2E). As such, we wanted to develop a purification protocol enabling high purity and improved yield. We introduced four alternative purification strategies based on namely, tandem sucrose cushion pelleting; tandem sucrose cushion pelleting with added Tween detergent in all purification steps; a two-step protocol relying on PEG precipitation and sucrose cushion pelleting (with Tween80); and finally a three-step protocol based on PEG

precipitation followed by gelatin chromatography and 30/50% sucrose cushion pelleting (with Tween80).

The tandem pelleting approach had higher virus yields compared to the three-step protocol, and significantly, the conventional “gold standard” gradient purification. The resulting yield was 83 times higher compared to the gradient purified virus (Figure 2E), however, the purity (66%) was not as good as in three-step protocol (99%). In addition, the tandem pelleting protocol is labor demanding particularly in laboratories lacking large-scale centrifuges. In comparison, while quicker to perform, the PEG precipitation protocol (CVB1#3b) resulted in a loss of virus (15% recovery). Nevertheless, PEG precipitation improved virus purity, and the three-step protocol yielded 19 times more virus than the traditional gradient purification method, with equal purity. Since the developed protocols provide good (tandem pelleting) or high (three step protocol) purity and improved yields over standard gradient purification protocol, their selection can be determined based on the needs of each laboratory and equipment available. Our protocols offer a quicker streamlined method to produce new enterovirus vaccines for preclinical testing in animal models.

Interestingly, formalin inactivation used in inactivated poliovirus vaccines caused a dramatic change in virus integrity. Following inactivation, only 5% virus protein was left in the vaccine preparation and most virus particles dispersed into smaller units, as seen with TEM (Figure 4D). This underlines the importance of properly analyzing vaccine preparations. It also indicates the poliovirus based inactivation protocol may not be suitable for other enteroviruses and virus-specific protocols may be required [36]. However, regardless of the concentration decrease, formalin-inactivated CVB1 still induced a strong virus-neutralizing antibody response in mice and protected against CVB1 infection *in vivo*.

Future work must develop more efficient concentration and purification methods suitable for an industrial scale, based, for example, on tangential flow filtration and chromatography [34]. To produce sufficient vaccine for future studies, optimization of the CVB1 inactivation step is also necessary.

5. Conclusion

In conclusion, we present an efficient and scalable CVB1 production protocol and purification process that is suitable for vaccine production. A three-step purification procedure can be scaled-up to purify several liters of virus-containing supernatant and allows for rapid progress to animal vaccination studies. For maximal virus yield an alternative tandem cushion pelleting protocol was also developed. The non-adjuvanted formalin inactivated CVB1 vaccine induced a strong, virus-neutralizing antibody response in vaccinated mice, providing valuable information for the development of new enterovirus vaccines for human use.

Figure legends

Fig. 1. Schematic showing production and purification protocols used.

Five different protocols were compared for purification of CVB1 (CVB1#1-4) and the most efficient protocol (three-step protocol) was tested also for CVB6.

Fig. 2. Characterization of CVB1 and CVB6 purified with different methods.

(A) Analysis of CVB1 total protein content (5 µl/well) on SDS-PAGE gel (B) and by Western blotting (1 µl/well) of VP1, stained with 5-D8/1. (C) Analysis of CVB6 total protein content on SDS-PAGE gel followed by (D) Western blotting analysis for the purified virus using pAb 1685 recognizing VP1. (E) Virus yield comparison (concentration and infectivity titer) expressed as normalized yield compared with the virus yield from CVB1#1. (F) Dynamic light scattering (DLS) analysis of CVB1#1-#4, CVB6 and PBS-0.1% Tween80 buffer without virus. The boxed-in area represents the virus peaks for CVB1 and CVB6. PBS-0.1% Tween80 contained 100% particles (determined by particle volume) with a

hydrodynamic diameter 10.7 nm representing Tween80 micelles. (G) The average sizes and volume distributions of the virus particles.

Fig. 3. The stability profiles of different CVB1 batches stored at 4°C for one week analyzed by dynamic light scattering (DLS).

The average sizes of the most predominant particles in the population were calculated from DLS measurements. (A) Purified CVB1 virus in R-buffer contained 85.0% and 91.2% particles (determined by particle volume) with a hydrodynamic diameter of 37.5 nm and 34.4 nm at days 0 and 7 respectively. (B) Purified CVB1 virus in citrate buffer contained 81.7% and 98.1% particles with a hydrodynamic diameter of 32.9 nm and 52.6 nm at days 0 and 7 respectively. (C) Purified CVB1 virus in PBS-Tween buffer contained 100% and 100% particles with a hydrodynamic diameter of 46.6 nm and 54.7 nm at days 0 and 7 respectively. (D) Purified CVB1 virus in M199-Tween buffer contained 99.5% and 100.0% particles (determined by particle volume) with a hydrodynamic diameter of 41.6 nm and 40.0 nm at days 0 and 7 respectively.

Fig. 4. Characterization of the CVB1#2 virus before and after formalin inactivation.

(A) DLS analysis of CVB1#2 before and after formalin inactivation. (B) SDS-PAGE and WB analysis of CVB1#2 before and after inactivation. (C) TEM analysis of CVB1#2 before formalin inactivation (bar: 500 nm from 15000 x magnification, bar: 50 nm from 30000 x magnification). (D) TEM analysis of CVB1#2 after formalin inactivation (bar: 500 nm from 20000 x magnification, bar: 50 nm from 30000 x magnification).

Fig. 5. Mice vaccinated with CVB1 vaccine are protected against CVB1 infection.

(A) Weights of mice after vaccination with 1 µg or 10 µg CVB1 vaccine. Black arrows indicate the days of vaccination. (B) Neutralizing antibody titres in mice vaccinated with 1 µg (n=4) or 10 µg (n=3)

of CVB1 vaccine ***p<0.001 compared to day 0 or as indicated (One-way ANOVA). (C) Weights of mice after infection with 10^6 PFU/mouse CVB1 in mice treated with 1 µg of vaccine, 10 µg of vaccine or control mice. (D) Viraemia on day 3 post infection or (E) replicating virus in the pancreas on day 5 post infection in control (n=4) or vaccinated mice receiving low (1 µg, n=4) or high (10 µg, n=3) CVB1 vaccine, as measured by standard plaque assay *p<0.05 compared to both low and high vaccine doses (One-way ANOVA). (F) VP1 positivity, as detected by immunohistochemistry in the pancreas of control mice or vaccinated mice on day 5 post infection (representative images) 16X magnification on the left and 40X magnification on the right.

Conflict of interest statement

The authors have no conflict of interest to declare. HH is a minor (5%) shareholder and member of the board of Vactech Ltd, which develops vaccines against picornaviruses.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version

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Tables

Table 1. Neutralizing antibody titers analyzed by plaque neutralization assay.

Vaccination group	Neutralizing titer				
CVB1 vaccine	**Day 0**	**Day 21**	**Day 35**	**Day 42**	**Day 49**
Mouse 1	**-**	**64**	**1024**	**1024**	**1024**
Mouse 2	**16**	**64**	**2048**	**2048**	**2048**
Negative control					
Mouse 3	**-**	**4**	**-**	**-**	**-**
Mouse 4	**-**	**-**	**4**	**-**	**-**

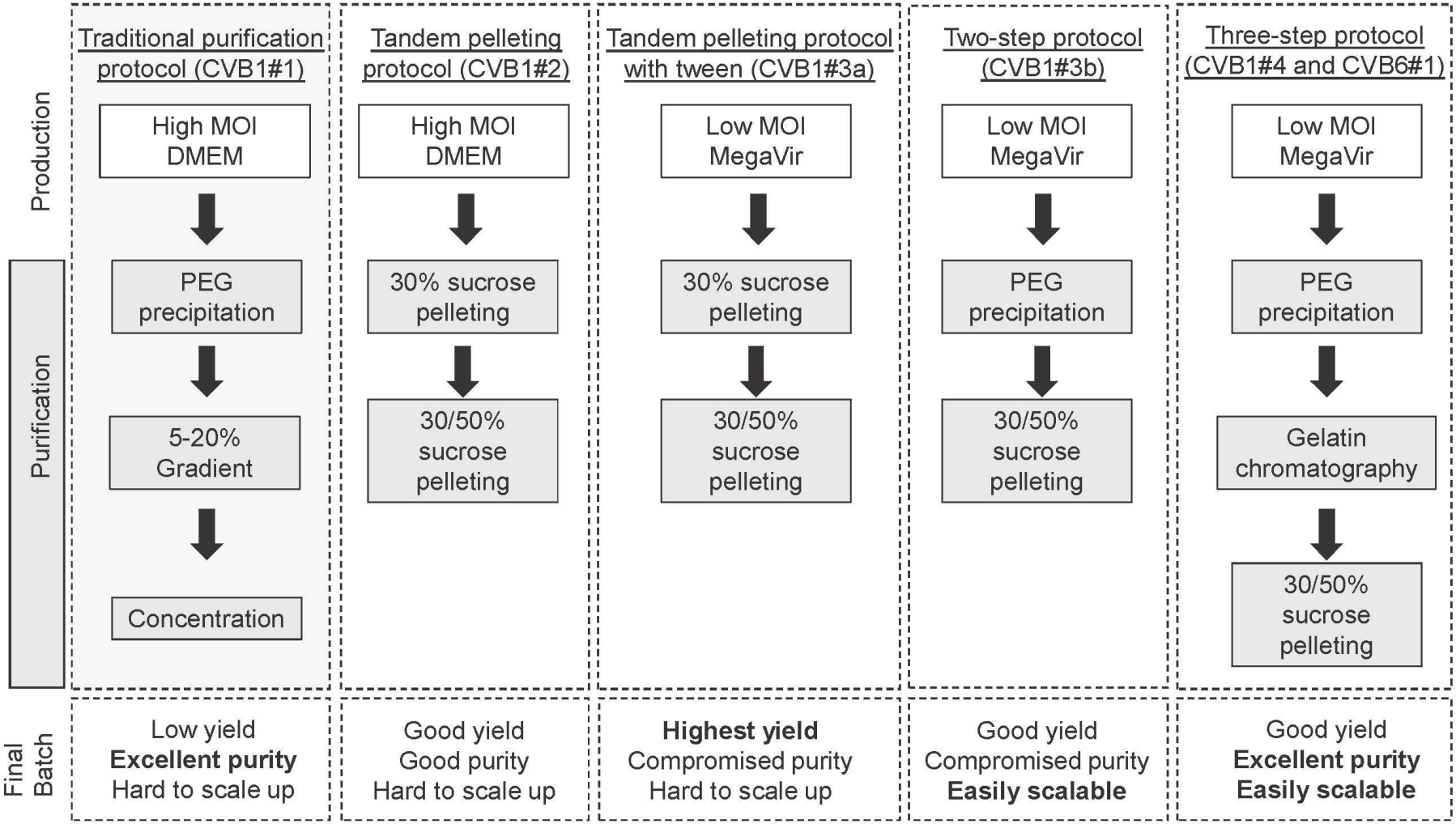
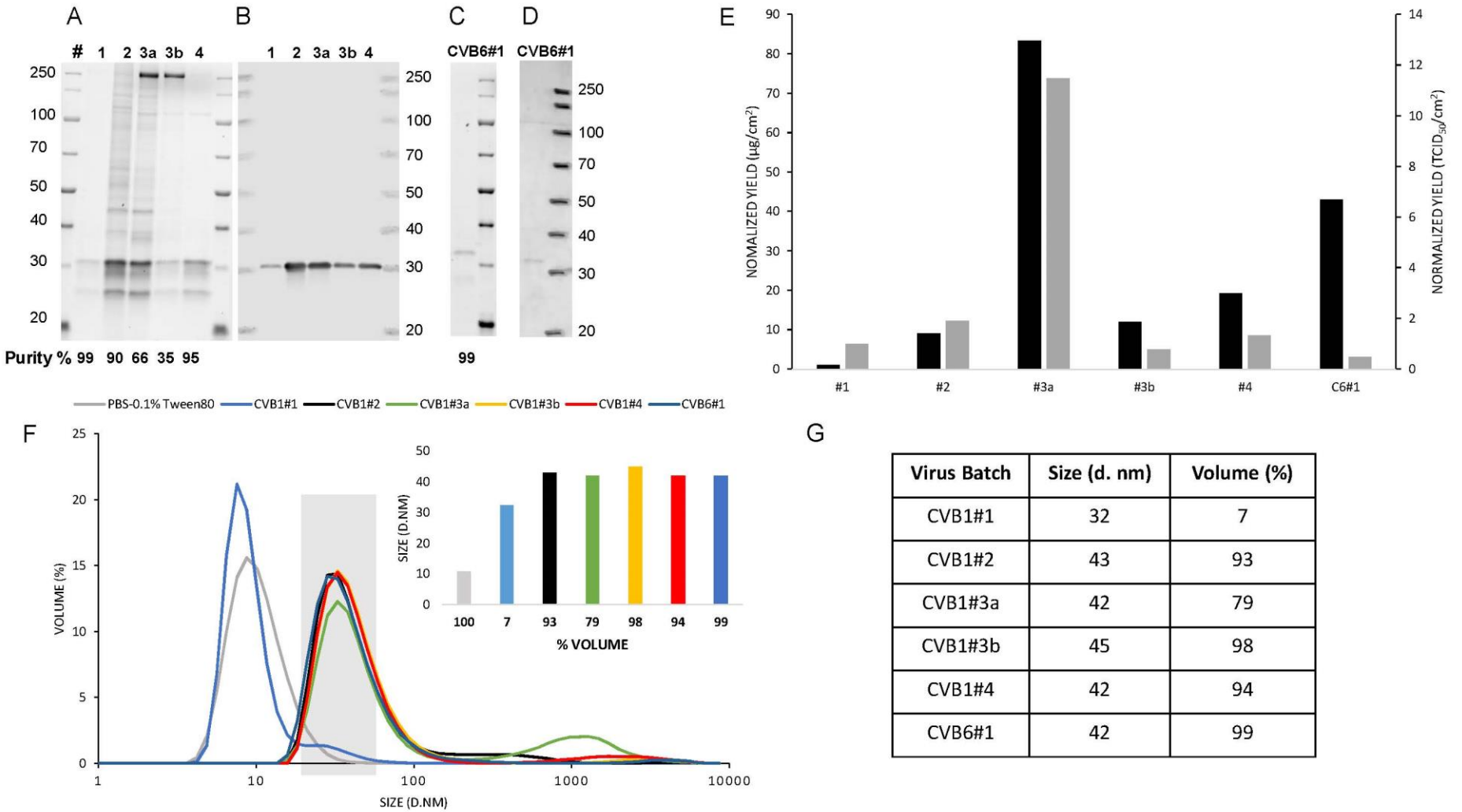


Fig. 1. Schematic showing production and purification protocols used. Five different protocols were compared for purification of CVB1 (CVB1#1–4) and the most efficient protocol (three-step protocol) was tested also for CVB6.



Virus Batch	Size (d. nm)	Volume (%)
CVB1#1	32	7
CVB1#2	43	93
CVB1#3a	42	79
CVB1#3b	45	98
CVB1#4	42	94
CVB6#1	42	99

Fig. 2. Characterization of CVB1 and CVB6 purified with different methods. (A) Analysis of CVB1 total protein content (5 ml/well) on SDS-PAGE gel (B) and by Western blotting (1 ml/well) of VP1, stained with 5-D8/1. (C) Analysis of CVB6 total protein content on SDS-PAGE gel followed by (D) Western blotting analysis for the purified virus using pAb 1685 recognizing VP1. (E) Virus yield comparison (concentration and infectivity titer) expressed as normalized yield compared with the virus yield from CVB1#1. (F) Dynamic light scattering (DLS) analysis of CVB1#1-#4, CVB6 and PBS-0.1% Tween80 buffer without virus. The boxed-in area represents the virus peaks for CVB1 and CVB6. PBS-0.1% Tween80 contained 100% particles (determined by particle volume) with a hydrodynamic diameter 10.7 nm representing Tween80 micelles. (G) The average sizes and volume distributions of the virus particles.

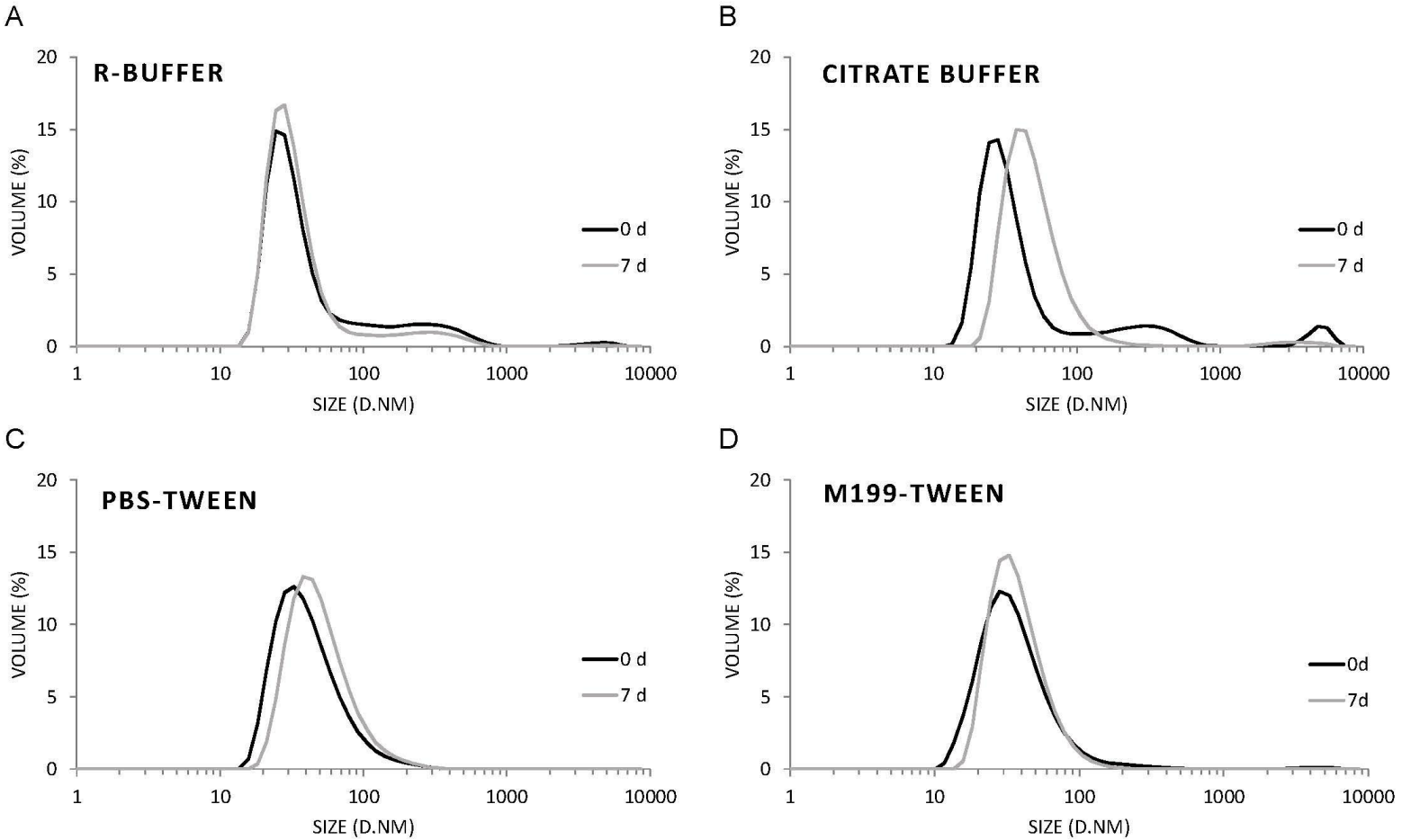


Fig. 3. The stability profiles of different CVB1 batches stored at 4 C for one week analyzed by dynamic light scattering (DLS). The average sizes of the most predominant particles in the population were calculated from DLS measurements. (A) Purified CVB1 virus in R-buffer contained 85.0% and 91.2% particles (determined by particle volume) with a hydrodynamic diameter of 37.5 nm and 34.4 nm at days 0 and 7 respectively. (B) Purified CVB1 virus in citrate buffer contained 81.7% and 98.1% particles with a hydrodynamic diameter of 32.9 nm and 52.6 nm at days 0 and 7 respectively. (C) Purified CVB1 virus in PBS-Tween buffer contained 100% and 100% particles with a hydrodynamic diameter of 46.6 nm and 54.7 nm at days 0 and 7 respectively. (D) Purified CVB1 virus in M199-Tween buffer contained 99.5% and 100.0% particles (determined by particle volume) with a hydrodynamic diameter of 41.6 nm and 40.0 nm at days 0 and 7 respectively.

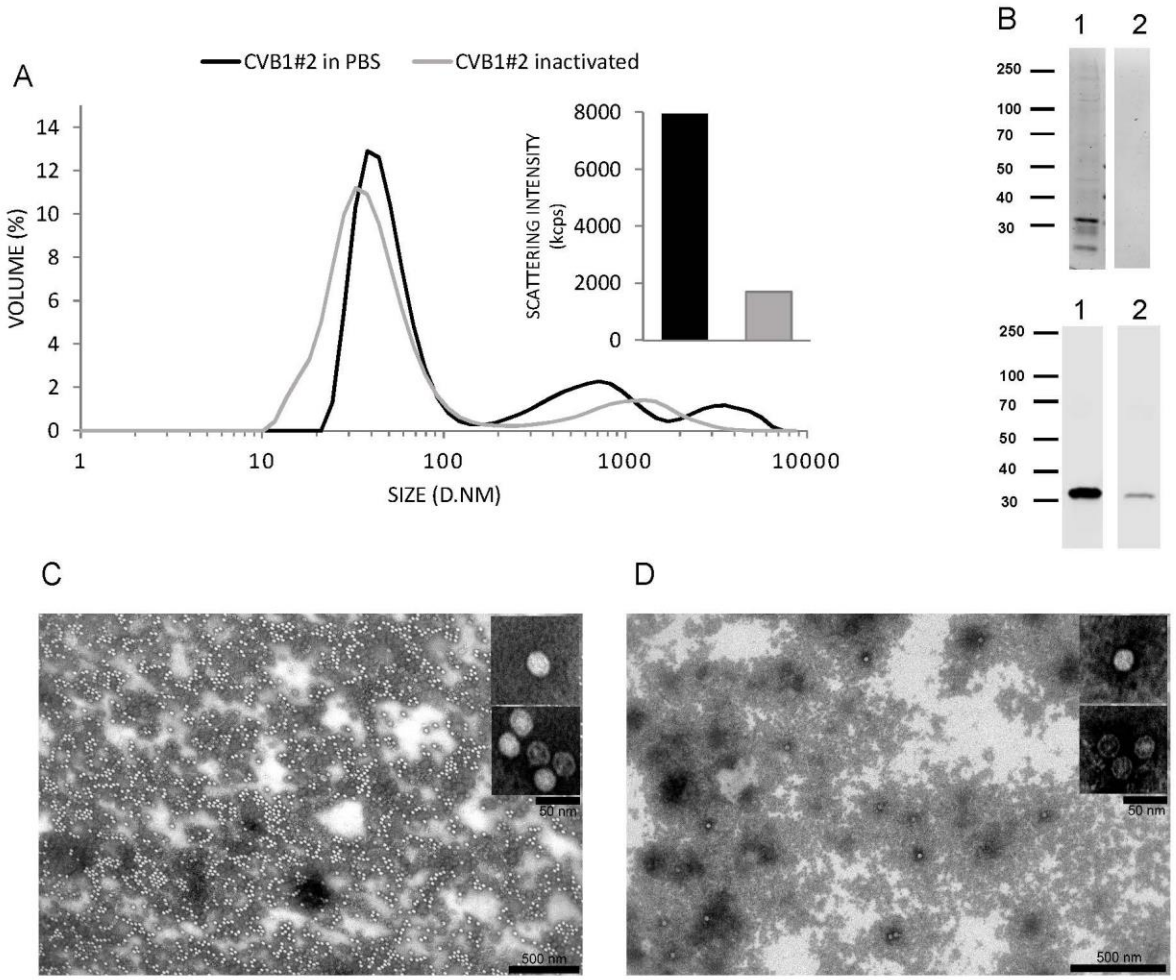


Fig. 4. Characterization of the CVB1#2 virus before and after formalin inactivation. (A) DLS analysis of CVB1#2 before and after formalin inactivation. (B) SDS-PAGE and WB analysis of CVB1#2 before and after inactivation. (C) TEM analysis of CVB1#2 before formalin inactivation (bar: 500 nm from 15,000 magnification, bar: 50 nm from 30,000 magnification). (D) TEM analysis of CVB1#2 after formalin inactivation (bar: 500 nm from 20,000 magnification, bar: 50 nm from 30,000 magnification).

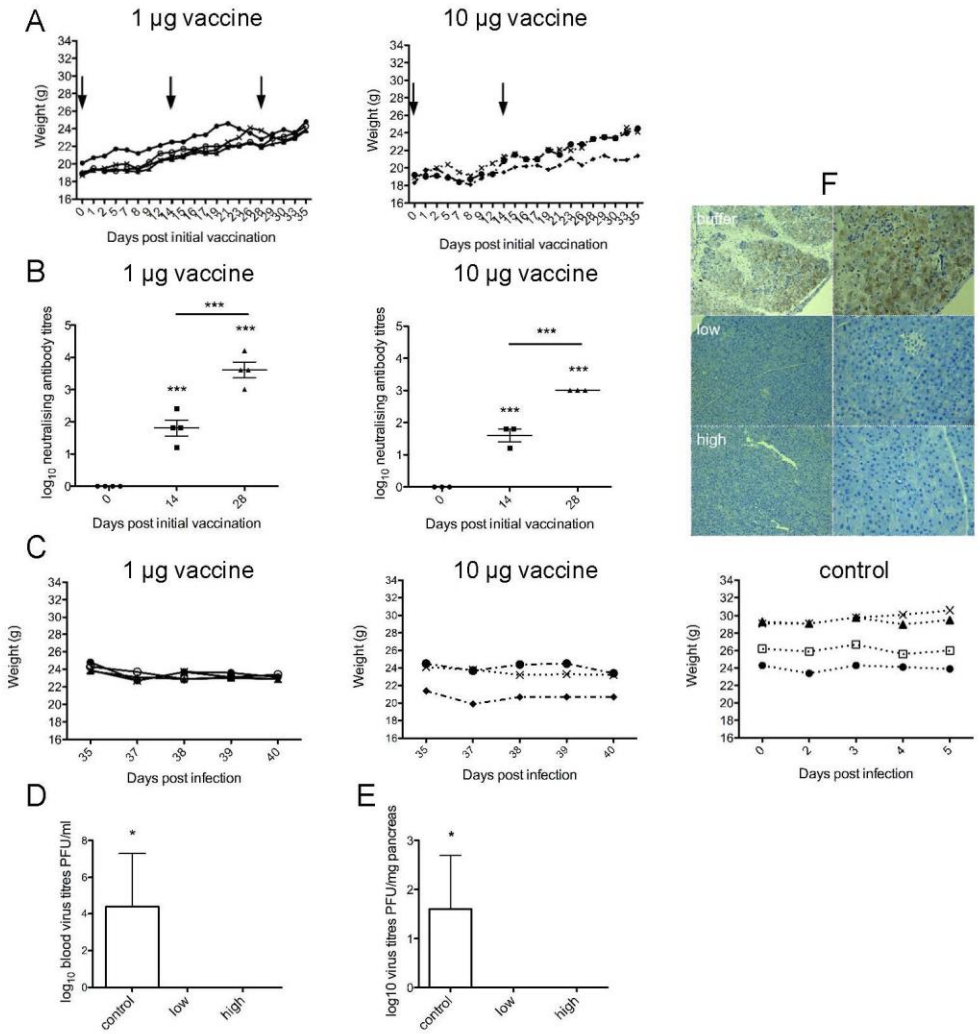


Fig. 5. Mice vaccinated with CVB1 vaccine are protected against CVB1 infection. (A) Weights of mice after vaccination with 1 mg or 10 mg CVB1 vaccine. Black arrows indicate the days of vaccination. (B) Weights of mice after infection with 106 PFU/mouse CVB1 after treatment with 1 mg or 10 mg of vaccine or vaccine buffer alone. (C) Neutralizing antibody titres in mice vaccinated with 1 mg (n = 4) or 10 mg (n = 3) of CVB1 vaccine ***$p < 0.001$ compared to day 0 or as indicated (One-way ANOVA). (D) Viraemia on day 3 post infection or (E) replicating virus in the pancreas on day 5 post infection in control (n = 4) or vaccinated mice receiving low (1 mg, n = 4) or high (10 mg, n = 3) CVB1 vaccine, as measured by standard plaque assay * $p < 0.05$ compared to both low and high vaccine doses (One-way ANOVA). (F) VP1 positivity, as detected by immunohistochemistry in the pancreas of control mice or vaccinated mice on day 5 post infection (representative images) 16 magnification on the left and 40 magnification on the right.

SUPPLEMENTARY MATERIAL

Supplementary Table 1. Details of CVB1 and CVB6 culturing and harvesting conditions.

<table>
<tr><th>Virus Batch</th><th>MOI</th><th>Infection (days)</th><th>Production medium</th><th>Additives at harvest (vol/vol%)</th><th>Virus release</th><th>Virus collection</th><th>Clarification</th></tr>
<tr><td>CVB1#1</td><td rowspan="2">5</td><td rowspan="2">2</td><td rowspan="2">DMEM</td><td>0.1% Tween80</td><td rowspan="6">3 x freeze-thaw</td><td rowspan="6">9610 x g, 4°C, 20 min</td><td rowspan="6">0.45 µm and 0.2 µm vacuum filtration</td></tr>
<tr><td>CVB1#2</td><td>0.3% DOC[1], 0.6% NP-40[2]</td></tr>
<tr><td>CVB1#3a</td><td rowspan="3">0.01</td><td rowspan="3">7</td><td rowspan="3">Megavir</td><td rowspan="3">0.1% Tween80</td></tr>
<tr><td>CVB1#3b</td></tr>
<tr><td>CVB1#4</td></tr>
<tr><td>CVB6#1</td><td>0.5</td><td></td><td></td><td></td><td></td><td></td></tr>
</table>

[1]Sodium Deoxycholate (DOC)
[2]Nonidet(N)P-40

Supplementary Table 2. Details of CVB1 and CVB6 purification protocols.

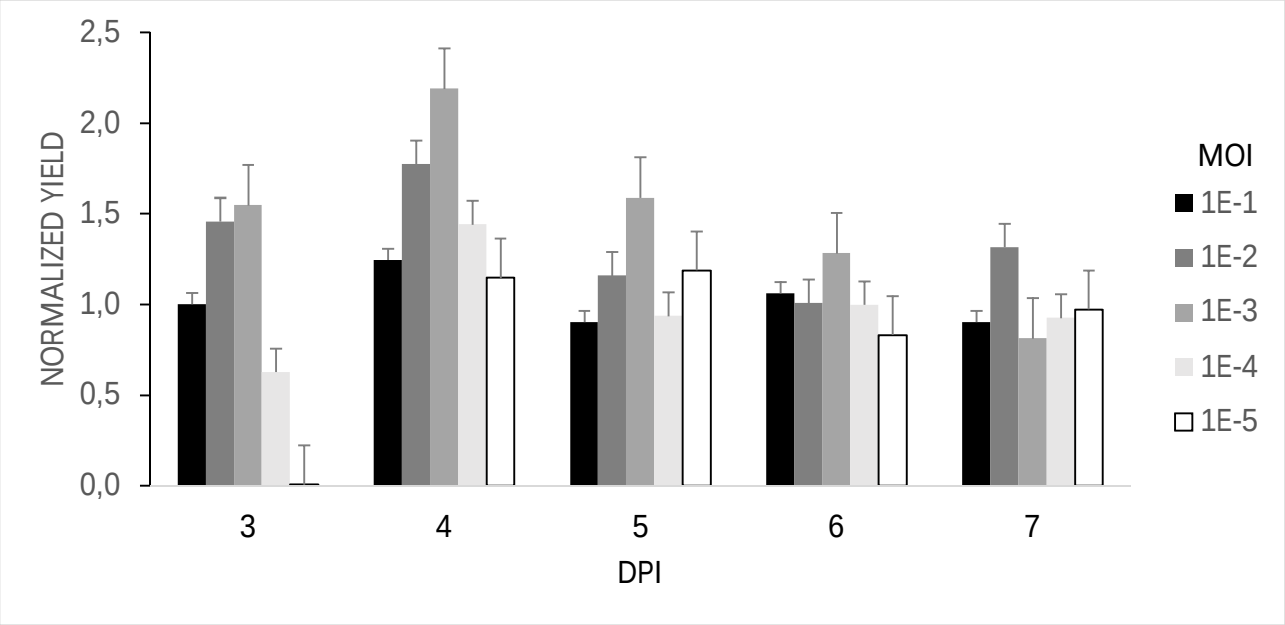
<table>
<tr><th>Virus Batch</th><th>Concentration or purification step</th><th>Solubilization</th><th>Purification step</th><th>Purification step</th><th>Solubilization</th></tr>
<tr><td>CVB1#1</td><td>PEG 6000[1]</td><td>PBS-0.1% Tween80</td><td>Discontinuous 5-20% sucrose gradient in PBS-0.1% Tween80, ultracentrifugation (103.9 k g, 4 h at 4°C)</td><td>Virus containing fractions were pooled and concentrated by pelleting using ultracentrifugation (108.6 k g, 4 h 4°C)</td><td>PBS-0.1% Tween 80</td></tr>
<tr><td>CVB1#2</td><td rowspan="3">Pelleting through 30% sucrose cushion using ultracentrifugation (175 k g, 14 h at 4°C)</td><td>R-buffer[2]</td><td rowspan="2">Pelleting through discontinuous 30/50% sucrose cushion by ultracentrifugation (285 k g, 14 h at 4°C)</td><td rowspan="2">-</td><td>R-buffer[2]</td></tr>
<tr><td>CVB1#3a</td><td rowspan="4">PBS-0.1% Tween80</td><td rowspan="4">M199-0.1% Tween80</td></tr>
<tr><td>CVB1#3b</td><td rowspan="3">PEG 6000[1]</td></tr>
<tr><td>CVB1#4</td><td rowspan="2">Incubation with gelatin sepharose (GE Healthcare #17-0956-01) rotation at 4°C for 16 h, followed by passing the collected supernatant through gelatin sepharose column</td><td rowspan="2">Pelleting through discontinuous 30/50% sucrose cushion by ultracentrifugation (285 k g, 14 h at 4°C)</td></tr>
<tr><td>CVB6#1</td></tr>
</table>

[1]Polyethylene Glycol (PEG) 6000

[2] R-buffer composition (10 mM Tris, 200 mM NaCl, 50 mM $MgCl_2$)

Suplementary Figure 1. The effect of MOI and culture time for CVB1 production.

In order to optimize the virus cultivation process in Vero-cells, the effect of MOI and culture time was studied by infecting them in 6-well plates with MOIs: 0.1, 0.01, 0.001, 0.0001 and 0.00001. The CVB1 productions were compared at days 3 – 7. The lowest yield was achieved with MOI 0.00001 at day 3 and the highest yield with MOI 0.001 at day 4.



Supplementary Table 3. The Vero cell residual DNA quantity of the purified viruses.

Virus batch	DNA (ng/µl)
CVB1#1	0.54 ± 0.36
CVB1#2	0.0068 ± 0.0030
CVB1#3a	0.146 ± 0.088
CVB1#3b	0.259 ± 0.164
CVB1#4	0.0005 ± 0.0004
CVB6#1	0.034 ± 0.025